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Heterogeneity in the Rate of Benzo[a]pyrene Metabolism in Single Cells: Quantitation Using Flow Cytometry

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We describe a method for quantitating heterogeneity in the rate of benzo[a]pyrene metabolism in single cells by using flow cytometry. We have used the technique to study the response of Hepa-1c1c7 mouse hepatoma cells to the microsomal enzyme inducer 2,3,7,8-tetrachlorodibenzo-p-dioxin. Cells responded in a relatively homogeneous fashion at different times of induction with a maximally inducing concentration of the inducer. However, the induction response could be heterogeneous at a submaximal inducer concentration. We found even higher heterogeneity of enzyme activity among low-activity variants derived from the Hepa-1c1c7 cell line. When cells of either high or low activity were isolated from such a clonal population, propagated, and reanalyzed, they displayed average enzyme activity and heterogeneity identical to the parental cells; therefore, the heterogeneity represents transient, nonheritable differences between cells within the population.

Measurement of microsomal drug-metabolizing enzyme activity in a given tissue or cell line usually requires 10^4 to 10^6 cells; thus, the observed value reflects an activity averaged over a large number of cells. However, cells of a given type may be heterogeneous with respect to their ability to metabolize foreign compounds. For example, subpopulations of rodent hepatocytes differ (i) in their response to the microsomal enzyme inducer phenobarbital (11, 12), (ii) in their content of smooth endoplasmic reticulum (9, 22), (iii) in their content of several forms of cytochrome p_{450} (3), (iv) in their susceptibility to toxicity mediated by reactive metabolic intermediates (7, 19), and (v) in their ability to oxidatively metabolize polycyclic aromatic hydrocarbons (24). We observed previously that subclones derived from the same clonal parental population of rat liver cells exhibited substantial variation in their ability to metabolize the environmental carcinogen benzo[a]pyrene (BP), as measured by the aryl hydrocarbon hydroxylase (AHH) assay. These and other subcloning experiments show that clonal cell lines can be heterogeneous with respect to AHH activity (14, 25). Heterogeneity in drug-metabolizing enzyme activity may complicate the interpretation of certain types of experiments, such as genetic analyses involving cell fusion or experiments involving drug-mediated cytotoxicity or neoplastic transformation. Studies of the microsomal metabolism of the polycyclic aromatic hydrocarbon BP illustrate the diversity of pathways by which xenobiotics can be metabolized, revealing a number of steps at which heterogeneity could occur (10).

We have described an assay of overall BP metabolism in which we measure the rate of disappearance of BP fluorescence from intact, viable cells. We have shown that the disappearance of BP fluorescence is due to metabolism of the carcinogen. We have used the fluorescence-activated cell sorter (FACS) in conjunction with the BP disappearance assay to analyze BP metabolism at the single-cell level and to isolate both high- and low-activity variants in BP metabolism from the Hepa-1c1c7 mouse hepatoma cell line (18).

Here, we describe a flow cytometric method for quantitating heterogeneity in overall BP-metabolizing activity among the cells of a given population. Using the Hepa-1c1c7 clonal cell line, which has been shown by subclonal analysis to be homogeneous (13), we have asked two questions related to the induction of BP-metabolizing activity by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD): first, whether all cells within a given population are equally responsive to different concentrations of inducer, and second, whether the kinetics of the induction response at a given inducer concentration are the same for all cells. In addition, we have used our technique to assess heterogeneity within variant cell populations which have decreased ability to metabolize BP.

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MATERIALS AND METHODS

Materials were obtained as follows: cell culture media, Dulbecco phosphate-buffered saline without calcium and magnesium (PBS), and trypsin-EDTA from GIBCO Laboratories (Grand Island, N.Y.); fetal bovine serum from Irvine Scientific (Irvine, Calif.) cell culture plasticware from Falcon Plastics (Oxnard, Calif.); BP from Aldrich Chemical Co. (Milwaukee, Wis.); 3-hydroxybenzo[*a*]pyrene (3-OHBP) and TCDD through the Cancer Research Program of the National Cancer Institute, Bethesda, Md.; NADPH from Calbiochem (La Jolla, Calif.).

Cells. The Hepa-1c1c7 cell line, which was derived from the Hepa-1 cell line (6) by subcloning (13), was the gift of Oliver Hankinson of the University of California at Los Angeles. Low-activity variants used in this study were isolated from the Hepa-1c1c7 cell line by selection in medium containing BP, as described by Hankinson (13). Such variants have an apparent mutational origin (13). Since the frequency of variants in the parental population is very low, we used the FACS to enrich for low-activity variants before selection in BP (18).

Cell culture. Cells were maintained as monolayers at 37°C and 5% CO_2 in α-minimal essential medium without nucleosides supplemented with 10% fetal calf serum. Trypsin-EDTA (0.05%–0.02%) was used to detach cells from dishes for subculture or fluorescence measurements. Cells were plated at 10^6 cells per 100-mm dish in 10 ml of medium 2 days before use. BP-metabolizing enzyme activity was induced by addition of TCDD in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in the medium was 0.1%; this had no detectable effect on cell viability or enzyme activity. Monolayers were trypsinized, diluted fivefold with PBS, centrifuged at 500 × g for 4 min and suspended in fresh PBS. After trypsinization, cells were kept on ice until assayed.

BP metabolism assay. A sample containing 2.5×10^5 cells per ml was incubated at 37°C in PBS containing 25 nM BP, and the disappearance of BP fluorescence was measured by flow cytometry. This incubation mixture was prepared by adding BP (in acetone) to PBS to make a 50 nM solution, followed by addition of an equal volume of cells at 5×10^5 cells per ml. Immediately after addition of the cells, the solution was mixed by rapid pipetting. This mixing procedure was adopted to achieve a uniform distribution of BP among the cells. The final concentration of acetone was 0.1%; this had no detectable effect on BP metabolism.

Flow cytometric analysis. Single-cell BP fluorescence was measured on a Becton-Dickinson FACS (1, 8, 15, 17). An argon laser provided mixed 350/360-nm excitation to the cell stream. BP fluorescence was measured by using a 10-nm bandpass filter with a center frequency of 405 nm (Ditric Optics, Hudson, Mass.). Cells were kept at 37°C during analysis. Cells take up BP in direct proportion to cell size, as measured in the scatter channel of the FACS (unpublished data). Therefore, to minimize the variation in cell fluorescence due to differences in cell size, we analyzed only those cells whose size differed by no more than 10% from the average cell size of the population. Narrowing the size gate further does not substantially decrease the fluorescence variation, but does increase the time necessary to analyze a given number of cells. A more useful tactic would be to express fluorescence measurements in terms of fluorescence divided by cell size, but this is technically impossible on our instrument. Typically, 5,000 cells were analyzed for each histogram. The ordinate indicates the percentage of total cells per fluorescence interval; each fluorescence interval on the abcissa represents one one-hundredth of a logarithmic cycle.

AHH assay. AHH was assayed by the method of Nebert and Gelboin (20). The fluorescence of alkali-extractable metabolites of BP was compared to that of a 3-OHBP standard, and phenol production is expressed in terms of 3-OHBP fluorescence equivalents.

RESULTS

Flow cytometry data. Flow cytometry allows the rapid (5,000 cells per s) measurement of BP fluorescence in individual cells from a population. These measurements are conveniently expressed as histograms, in which the number of cells is plotted as a function of fluorescence. We measured BP metabolism by incubating cells with the carcinogen and following changes in the fluorescence histograms with time. BP is very hydrophobic and rapidly partitions into cells, so that soon (15 to 30 s) after exposure to the compound, all cells exhibited high fluorescence. With time, cells having relatively high BP-metabolizing activity lost fluorescence, which decreased toward the cellular background fluorescence level. In contrast, cells with relatively low BP-metabolizing activity lost fluorescence more slowly, and their fluorescence remained relatively high with time. We have previously shown that cells behave independently in such an assay; a change in the BP concentration of one cell does not influence the BP concentration of other cells in the same population (18).

Qualitatively, fluorescence histograms can suggest the presence of heterogeneity in BP-metabolizing activity among cells within a population. For example, we grew together as a mixed culture wild-type Hepa-1c1c7 cells (which have high inducible BP-metabolizing activity) and variant BP-resistant (BP^r) cells (which have undetectable BP-metabolizing activity) isolated from the Hepa-1c1c7 line. The cells were induced, suspended, incubated with BP, and analyzed for fluorescence with the FACS. The fluorescence histogram obtained after a 20-min incubation with BP revealed two distinct peaks (Fig. 1A), confirming the presence of heterogeneity in BP-metabolizing activity within the mixed cell population. However, other types of experiments can generate qualitatively similar fluorescence histograms. For example, we incubated Hepa-1c1c7 cells, which have been shown to be homogeneous by subclonal analysis (13), for 18 h with a low (3 pM) concentration of the microsomal enzyme inducer TCDD. The fluo-

rescence histogram obtained after a 20-min incubation with BP (Fig. 1B) was similar to that observed with the wild-type–variant mixture (Fig. 1A), suggesting the presence of heterogeneity in BP-metabolizing activity within this (presumably) homogeneous clonal cell line. Indeed, there appeared to be two distinct subpopulations of cells. To better understand this result, and those of other experiments which were suggestive of heterogeneity, we have developed a quantitative method for assessing the relative heterogeneity in BP-metabolizing activity among the individual cells of a given population.

Method for quantitation of heterogeneity. We have shown previously that the kinetics of BP metabolism, as measured by the disappearance of BP fluorescence, conform to the Michaelis-Menten equation:

$$-\frac{d[\mathrm{BP}]}{dt} = V = \frac{V_{\max}[\mathrm{BP}]}{K_m + [\mathrm{BP}]} \quad (1)$$

where V is the velocity of BP metabolism, [BP] is the concentration of BP, $V_{\max}$ is the maximal velocity, and K_m is the Michaelis-Menten constant (A. G. Miller and J. P. Whitlock, Jr., submitted for publication). Thus, any method for quantitating heterogeneity in BP metabolism must consider the non-linearity of BP metabolism with time under the conditions of the assay. BP metabolism cannot be made a linear function of time by simply increasing the BP concentration, because of the insolubility of BP.

We have approached the problem by considering the time taken by a given cell to reach an arbitrary BP concentration $[\mathrm{BP}]_a$ from the starting BP concentration $[\mathrm{BP}]_0$. We show below that this time, which we will call t_a, is inversely related to the $V_{\max}$ of the cell in question. From equation (1):

$$-\frac{K_m + [\mathrm{BP}]}{[\mathrm{BP}]} d[\mathrm{BP}] = V_{\max}\, dt \quad (2)$$

Integrating both sides of the equation:

$$K_m \ln [\mathrm{BP}] + [\mathrm{BP}] = -V_{\max}\, t + C_i \quad (3)$$

where C_i is a constant of integration. Given $[\mathrm{BP}]_0$ and $[\mathrm{BP}]_a$, we can solve this equation to obtain t_a:

$$V_{\max}\, t_a = K_m \ln \frac{[\mathrm{BP}]_0}{[\mathrm{BP}]_a} + [\mathrm{BP}]_0 - [\mathrm{BP}]_a = C \quad (4)$$

Since the right side of the equation is a constant, t_a is inversely proportional to the $V_{\max}$ of the cell in question. In other words, the time it takes a given cell to metabolize an arbitrary fraction of the BP it contains is inversely proportional to the $V_{\max}$ of the BP-metabolizing enzyme system of that cell.

Using the FACS, we cannot directly measure the time it takes each cell in a population to reach an arbitrary fluorescence, F_a, equivalent to a BP concentration of $[\mathrm{BP}]_a$. However, we can easily determine from the histograms the number of cells above (or below) an arbitrary F_a at any time after the addition of BP. Cells with fluorescence greater than F_a at time t have $V_{\max}$ values less than C/t, and cells with fluorescence less than F_a at time t have $V_{\max}$ values greater than C/t. The increase in the number of cells with fluorescence less than F_a in any time interval t_1 to t_2 is the number of cells with $V_{\max}$ values between C/t_1 and C/t_2. Since the FACS can rapidly analyze cells, a large number of histograms can be obtained in a short time (i.e., 30 min). We then analyze this data further by plotting as a function of time (and, therefore, $V_{\max}$) the fraction of cells having fluorescence greater than F_a. The slope of this plot reveals the heterogeneity in BP-metabolizing enzyme activity within the cell population. For example, if a cell population is homogeneous with respect to its ability to metabolize BP, then each cell within the population will reach F_a at the same time, and the slope of the above plot will be very steep. Conversely, if the cell population is heterogeneous in its BP-metabolizing activity, it will take longer for some cells within the population to reach F_a than for others; therefore, the slope of the above plot will be less steep. Thus, the steeper the slope, the less the heterogeneity.

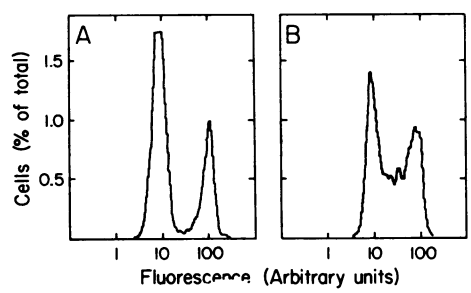

FIG. 1. Apparent heterogeneity in BP metabolism revealed by fluorescence histograms. (A) A mixture of 10^6 Hepa-1c1c7 (wild-type) and 10^6 BP^{r}c1 (low-activity variant) cells were grown together for 2 days before assay. The mixture was induced for 18 h with 1 nM TCDD and assayed for BP metabolism as described in the text. The fluorescence histogram obtained at 20 min after the addition of BP is shown. There are fewer cells in the high-fluorescence (low BP-metabolizing) peak due to the slower growth rate of BP^{r}c1 cells in comparison with Hepa-1c1c7. (B) Hepa-1c1c7 cells were induced with 3 pM TCDD for 18 h and analyzed for BP metabolism, as described in the text. The 20-min histogram is shown.

It is useful to plot the fraction of cells with fluorescence greater than F_a as a function of log t, rather than t, because: (i) a wider range of t (and V_{max}) can be displayed in a single plot; (ii) for the cells we have analyzed, single cell BP-metabolizing activity appears to be distributed log-normally; (iii) $\log t = \log C - \log V_{max}$, so V_{max} has the same scale (i.e., logarithmic) as t; (iv) when the data are plotted as a function of log V_{max}, percentage variations in V_{max} are represented equidistantly. Finally, it is also useful to plot the derivative of the slope with respect to time; this reveals the fraction of the cell population with a given V_{max}.

Analysis of heterogeneity. We have used this method to analyze cell populations known to differ in their ability to metabolize BP, as determined by the AHH assay. We induced the activity of the BP-metabolizing enzyme system in wild-type Hepa-1c1c7 cells by exposure to 1 nM TCDD for either 2 h (at which time the cells' average activity was 18 pmol of 3-OHBP per min per mg of protein) or 18 h (AHH activity = 100 pmol of 3-OHBP per min per mg of protein). Preliminary experiments showed that these induction protocols produced cell populations which were relatively homogeneous with respect to BP-metabolizing activity, and that our method of analysis should be able to resolve these populations from a mixture. We then analyzed these cell populations for heterogeneity, both separately and after mixing them together, using the approach described above.

Figure 2A shows the fraction of cells having BP fluorescence greater than an arbitrary F_a, plotted as a function of log t and log V_{max}. Both cell populations, when analyzed separately, exhibited similar, rather steep slopes; as expected, the population with the higher activity (cells induced for 18 h) lost fluorescence relatively rapidly, and the curve is to the left of the curve for the cells with lower activity. The derivatives of these curves indicate that these individual populations are somewhat heterogeneous with respect to their BP-metabolizing activity; in both cases, about 80% of the cells fall within a threefold range of V_{max}.

Analysis of these two cell populations after mixing yields a plot which is essentially the sum of the plots of the two populations analyzed separately. As expected, the derivative plot (Fig. 2B) reveals the presence of two distinct subpopulations of cells which differ in BP-metabolizing activity. Thus, these observations indicate that we are able to determine the degree of heterogeneity in BP-metabolizing activity within a cell population. We have used this approach to examine several aspects of the induction of BP-metabolizing activity in Hepa-1c1c7 cells.

In our experiments, we have used TCDD to induce BP-metabolizing activity because (i) the compound is metabolized very slowly, if at all, and therefore its concentration remains essentially constant during the induction period; and (ii) TCDD does not compete for the BP-metabolizing enzyme system, as do inducers such as benz[*a*]anthracene, 5,6-benzoflavone, and 7,8-benzoflavone (Miller and Whitlock, submitted for publication).

We first asked whether, at a maximal inducing concentration of TCDD, the wild-type cell population was heterogeneous with respect to the kinetics of induction of the BP-metabolizing enzyme system. We exposed cells to 1 nM TCDD for 2, 4, or 18 h (at which time the induction is maximal) and analyzed these cell populations for heterogeneity in BP metabolism

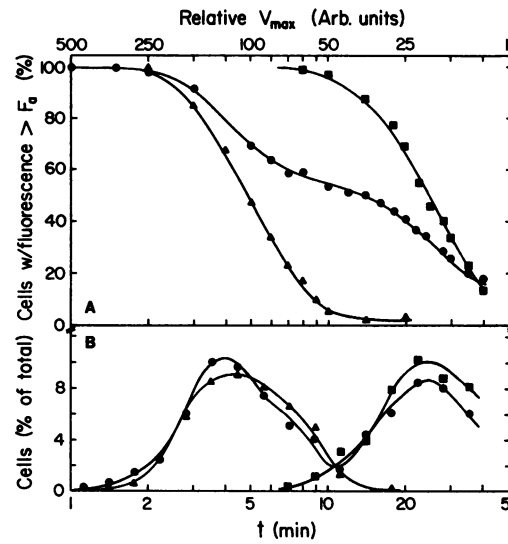

FIG. 2. Analysis by the new method of a known heterogeneous population. Wild-type Hepa-1c1c7 cells were induced with 1 nM TCDD for either 2 or 18 h. These cells, and a mixture of these cells, were analyzed for BP metabolism as described in the text. Fluorescence histograms obtained at the times indicated on the abcissa were analyzed as described in the text. (A) The percentage of cells with fluorescence greater than an arbitrary value F_a is plotted as a function of time. Symbols: (■) cells induced for 2 h; (▲) cells induced for 18 h; (●) mixture of cells induced for 2 and 18 h. The upper scale, indicating the relative V_{max}, was arbitrarily defined as $V_{max} = 500/t$. (B) Derivatives of the above curves are shown. Derivatives were obtained from the curves (not the data points) by dividing each logarithmic cycle into 10 linear units and determining the change in the percentage of the cells above F_a in each unit. These values are plotted in the center of each unit to obtain the derivative curves. The scale is expanded by a factor of two for the mixture since only half of the cells in the mixture are derived from the individual populations.

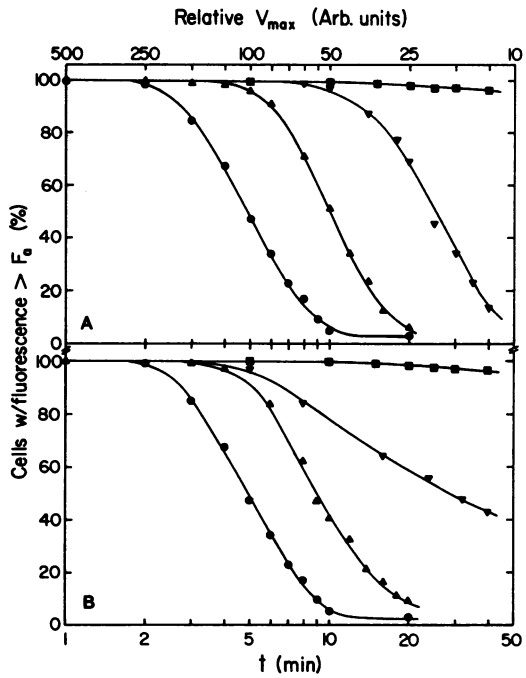

FIG. 3. Analysis of heterogeneity in the induction of BP-metabolizing enzyme activity. (A) Kinetics of induction. Hepa-1c1c7 cells were induced with 1 nM TCDD for 0 (■), 2 (▼), 4 (▲), or 18 h (●) and analyzed as described in the legend to Fig. 2 and in the text. (B) Response to submaximal concentrations of the inducer. Hepa-1c1c7 cells were induced for 18 h with TCDD at the indicated concentrations: (●) 1nM; (▲) 10 pM; (▼) 3 pM; (■) no TCDD. Cells were analyzed as described in the legend to Fig. 2 and in the text.

as described above. The results (Fig. 3A) indicate that the shape of the plot is very similar for all three conditions of induction; as expected, as induction proceeds and enzyme activity increases, the curve shifts to the left. These curves reveal no evidence for increased heterogeneity in BP-metabolizing enzyme activity early in the induction period. In each case, about 80% of the cells fell within a threefold range of V_{max}. Thus, these results suggest that, at maximal inducing concentrations of TCDD, the kinetics of induction of the BP-metabolizing enzyme system are similar among the individual members of the Hepa-1c1c7 cell population.

We also asked whether there was heterogeneity within the Hepa-1c1c7 cell population with respect to its response to submaximal concentrations of inducer. We exposed cells for 18 h to TCDD at concentrations of 1 nM, 10 pM, and 3 pM and analyzed these populations for heterogeneity in BP-metabolizing enzyme activity. The results (Fig. 3B) reveal that, at 1 nM and 10 pM TCDD, the shape of the curve is similar to that observed above (Fig. 2 and 3A), revealing no evidence for increased heterogeneity in the BP-metabolizing enzyme system at these concentrations of inducer. In contrast, the shape of the curve for cells exposed to 3 pM TCDD is quite different, with a more gentle slope, showing greater heterogeneity. If we assume that the curve is symmetrical (see Discussion), 80% of the cells fall within a ninefold range of V_{max}, as opposed to the threefold range observed previously. However, our analysis reveals that the cells are distributed rather uniformly over a broad range of enzyme activities and are apparently not divided into two distinct subpopulations, as individual histograms might suggest (Fig. 1B). The distribution in the histograms is explained by the fact that cells cannot display fluorescence less than background cell fluorescence. With time, cells tend to form a peak at the background cell fluorescence level, and thus may appear to be a separate subpopulation.

Table 1 shows the relative activities for BP metabolism, derived from the data in Fig. 3, for cell populations exposed to TCDD under various conditions. The median V_{max} for each population is proportional to its AHH activity, showing that our method of analysis yields results consistent with the standard AHH bulk assay.

We have isolated variants of the Hepa-1c1c7 cell line which have markedly reduced, inducible BP-metabolizing activity, as measured both by the AHH assay and by the BP disappearance assay. After inducing BP-metabolizing activity with TCDD (1 nM, 18 h), we analyzed four of these low-activity variants for heterogeneity in their ability to metabolize the carcinogen. The results (Fig. 4) show that one variant (BP^r c1) has undetectable activity, and that the other

TABLE 1. Comparison of enzyme activity measured by the AHH assay or derived by the new method of analysis[a]

Induction conditions		V_{max}	
TCDD (pM)	Time (h)	AHH (pmol of 3-OHBP per min per mg)	Flow cytometric analysis (arbitrary units)
0	—	3.7 ± 0.4	<12
3	18	20 ± 1	17
10	18	66 ± 1	61
1,000	18	100 ± 8	102
1,000	4	58 ± 2	54
1,000	2	18 ± 1	23

[a] After induction, AHH was determined as described in the text, and the relative median cell V_{max} was calculated by dividing 500 by the time taken for half the cells in a population to reach F_a. The number 500 was arbitrarily chosen so that AHH values would roughly match the relative median cell V_{max} values.

three populations are relatively heterogeneous in BP-metabolizing activity, when compared with wild-type cells. We wondered whether this heterogeneity might reflect the accumulation of revertants, since a small percentage of the BP^r cells have activity comparable to the average wild-type cell activity (Fig. 4). Therefore, using the FACS, we isolated relatively high-activity cells from two variant populations, propagated them for several weeks, and reanalyzed them for heterogeneity. Each isolate was indistinguishable from the parental variant population from which it was derived (Fig. 4). Thus, in this case, the heterogeneity in BP-metabolizing activity among the cells of a clonal line is not due to the presence of revertants.

In similar experiments, we sorted from the variant populations cells with relatively low activity, to determine whether we could isolate a low-activity subpopulation which did not contain high-activity cells. Again, the isolates contained as many high-activity cells as the parental population (data not shown). Thus, the heterogeneity is due to transient, nonheritable differences in enzyme activity among the cells of these low-activity variants. This heterogeneity appears to be an inherent property of these cells.

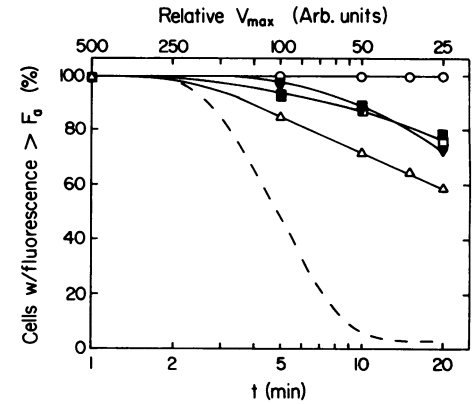

FIG. 4. Analysis of heterogeneity in BP metabolism in low-activity variants derived from Hepa-1c1c7 cells. Cells were induced with TCDD (1 nM, 18 h) and analyzed as described in the legend to Fig. 2 and in the text. Symbols: (○) BP^{r}c1; (▽) TAOc1BP^{r}c1; (□) BP^{r}c2TAO; (△) BP^{r}c6. The dashed line is an analysis of Hepa-1c1c7 cells under the same conditions. The designation BP^r indicates resistance to BP toxicity. The designation TAO indicates resistance to 6-thioguanine, 8-azaguanine, and ouabain. The FACS was used to select cells of high activity from two of these clones. The cells were grown and reanalyzed: (▼) cells from TAOc1BP^{r}c1; (■) cells from BP^{r}c2TAO.

DISCUSSION

We observed previously that diploid subclones isolated from a cloned line of Buffalo rat liver cells exhibited substantial (i.e., 10- to 20-fold) variation in both basal and induced AHH activity, showing that the parental population was heterogeneous with respect to its ability to metabolize BP (25). Hankinson performed similar cloning experiments using Hepa-1c1c7 cells, and the variation he observed in basal and induced AHH activities among the subclones could be entirely accounted for by experimental error, showing that, by this subcloning criterion, Hepa-1c1c7 cells are homogeneous with respect to BP-metabolizing activity (13). Our present studies illustrate an alternative approach for studying heterogeneity, employing the FACS to measure BP metabolism in single cells. It is much more rapid than subcloning, and generates a more representative result, since we can study many more cells in a given population. Our findings provide direct evidence that the individual members of a cloned cell population may exhibit heterogeneity in their ability to metabolize BP. Our studies of the low-activity variants show that the propensity to become heterogeneous with respect to BP metabolism is an inherent property of these cells and is not due to the accumulation of new variants. This heterogeneity develops rapidly and in the absence of any applied mutational stress.

The usefulness of this method for analyzing cells with low enzyme activity is limited by the length of time required for assay, both because of the expense involved in long FACS experiments and because of possible cellular changes during long incubations of the cells with BP. We can increase the sensitivity of the method somewhat by increasing the F_a used for analysis. To facilitate the comparison of data from different experiments described here, we have used the same relative F_a, about halfway (on a logarithmic fluorescence scale) between cells unlabeled or labeled with BP. The same histogram data, however, can be analyzed using lower or higher F_a values, with the result that curves showing percentage of cells above F_a versus time are shifted to the right or left, respectively (equation 4). For example, if the cells induced with 3 pM TCDD for 18 h (Fig. 3B) are analyzed using a higher F_a, 90% of the cells pass F_a in 40 min. The curve is symmetric, supporting the assumption made earlier (see Results).

Factors unrelated to enzyme activity contribute to heterogeneity which we observe at the single-cell level. We can estimate their contribution from the observation that all cells examined here exhibited a narrow fluorescence distribution soon (e.g., 30 s) after addition of BP; at this time, 80% of the cells fell within a 1.5- to 1.7-fold range in fluorescence. If these cells were to lose BP fluorescence at exactly the same rate, our

analysis would show an apparent 1.4- to 2.0-fold range in V_{max}, assuming the same F_a used in our experiments. Thus measurement errors introduce an apparent variation in V_{max} of about 1.7-fold. Assuming log-normal distributions, the logarithm of the fold variation is proportional to the standard deviation of the distribution. The variance of the error can be subtracted from the measured heterogeneity to obtain a better estimate of the true heterogeneity. Thus, a measured heterogeneity in V_{max} of 3-fold reflects a true heterogeneity of 2.6-fold, and a measured 9-fold heterogeneity reflects a true heterogeneity of 8.4-fold.

We have used cytometric data to calculate relative V_{max} values. In principle, by knowing the cell volume and the partition coefficient of BP between cells and the medium, we could calculate absolute enzyme activities of these cells directly from our data (Miller and Whitlock, submitted for publication). In practice, however, the usefulness of our method lies in its ability to quantitate heterogeneity in BP metabolism within a cell population. This approach should also be applicable to other enzymes which can be studied by flow cytometry, provided that the relationship in equation (4) applies for the enzyme involved. For instance, in a product appearance assay using saturating substrate concentrations, the rate of product appearance is $V_{max}t$. So the time taken for a given cell to reach a given fluorescence is inversely related to the V_{max} of the cell. Equation (4) of our derivation is the same result derived by Benditt and Arase (5) in their analysis of enzyme kinetics in a histochemical system. In their derivation, they implicitly assume that the substrate concentration is constant during metabolism, even though the enzyme is not saturated. For systems in which this condition applies, our method of analysis will also be useful.

We observe marked heterogeneity of enzyme activity in some cells, especially the BP^r variants. The only obvious transient difference among cells in these populations is their position in the cell cycle. Before trypsinization for assay, the cells are in logarithmic growth, and thus representatives from all positions of the cell cycle are present. TCDD has no effect on the growth rate of cultured cells (16), so this situation applies to induced as well as uninduced cells. Synchronously grown cultures of mouse liver cells display much greater AHH induction during the S phase of the cell cycle (4). Thus, the wide variation in enzyme activity that we observe may relate to the distribution of cells in various parts of the cell cycle. DNA fluorochromes exist which can be excited by using the same wavelength as BP (17) but have different emission wavelengths; thus, it may be possible to directly investigate the relation of cell cycle to BP-metabolizing activity by using the FACS.

We cannot provide a simple explanation for our observation that Hepa-1c1c7 cells respond relatively heterogeneously to a low (3 pM) concentration of TCDD, while responding relatively homogeneously to half-maximal (10 pM) and maximal (1 nM) concentrations of the inducer. We considered the possibility that the heterogeneous response to induction with 3 pM TCDD might be an artifact related to uneven distribution of TCDD among the members of the cell population. However, the same heterogeneous response occurs no matter how the inducer is added to the cells. In addition, this heterogeneity does not appear to be an artifact related to the relatively low BP-metabolizing activity of the cells, because other cell populations with similarly low activity (e.g., following a 2-h exposure to 1 nM TCDD) do not exhibit a similar degree of heterogeneity.

Clonal variation has been observed previously not only for AHH activity (14, 25), but for albumin production in rat hepatoma cells (21), tyrosine aminotransferase activity in hepatoma tissue culture cells (2), and hypoxanthine-guanine phosphoribosyl transferase activity in mouse L cells (23); thus, heterogeneity within populations of cultured cells is not unique to the BP-metabolizing enzyme system. Here, we show that even populations which are apparently homogeneous by the clonal assay may be heterogeneous when studied at the single-cell level. The possibility of cell heterogeneity complicates the interpretation of experiments which employ bulk enzyme assays to assess the susceptibility of cell populations to the toxic or carcinogenic effects of BP. Thus, in studies of the metabolic activating and detoxifying pathways for BP and other foreign compounds, it may be important to know not only the average activity of a given enzyme, but also the spread of activities which that average reflects. In addition, these experiments provide important clues for an understanding of the regulation of the polycyclic aromatic hydrocarbon-inducible cytochrome p_{450} enzyme system.

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